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Soybean meal level and probiotics in first feeding fry diets alter the ability of rainbow trout *Oncorhynchus mykiss* to utilize high levels of soybean meal during grow-out

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ABSTRACT

Inclusion rates of soybean meal in salmonid diets are currently kept low to minimize detrimental effects on growth, enteritis and immune responses. Probiotics have been used to treat both infectious and noninfectious enteritis in humans and other terrestrial animals and may represent a feasible method for increasing soy utilization in soy-sensitive aquatic species. To test the hypothesis that probiotics incorporation in rainbow trout starter diets can induce immune-mediated soybean tolerance, a two-phase experimental design was employed. In the starter phase (first feeding, 0.13 ± 0.01 to 6.5 ± 0.32 g fish^{-1}), a practical-type diet was formulated to contain 48% crude protein and 20% crude fat containing either 0 (S0), 10 (S10) or 20% (S20) soybean meal (SBM) and supplemented with (S0P, S10P, S20P) or without a commercially available probiotic (Mycolactor Dry Probiotic®) in a 3×2 factorial design. Diets were fed to four replicate tanks of fish per treatment (300 fish tank^{-1}; House Creek strain) for 8 weeks. Trout were reared in 150 L tanks supplied with 4 L min^{-1} of constant temperature (14.8 °C) flow-through spring water. Potentially soy tolerant rainbow trout produced by feeding probiotics and increased levels of soybean meal in starter diets as described above were then fed the industry standard level 15% (G15) or a diet with a challenge level of 43% (G43) of soybean meal during a 12 week grow-out and digestibility trial. Pathological changes were observed in intestines of fish fed the 43% SBM during grow-out; however, these changes were less severe when fish had been exposed to soybean meal in starter diets. The addition of probiotics to starter diets appeared to improve soybean meal utilization by first feeding rainbow trout, but probiotic use had only limited benefits when they were not continuously provided in the diet.

1. Introduction

Increased utilization of plant protein meals has been embraced as a sustainable alternative to fish meal, with soybean meal most commonly used (Nordrum et al., 2000). Soybeans have a high content of available protein with a fairly well-balanced amino acid profile; however, inclusion rates are currently kept low to minimize detrimental effects on growth and immune response commonly associated with anti-nutritional factors. Inclusion rates in the diet of rainbow trout are currently less than 20% (Hardy, 2002). At levels of >20% reduced weight gain and feed efficiency (Olli and Krogdahl, 1995; Olli et al., 1995; Rumsey et al., 1994) and pathomorphological changes in the distal intestinal epithelium with diarrhea (Van den Ing et al., 1991; Rumsey et al., 1994) are observed. These intestinal changes are most correctly described as non-infectious, subacute enteritis (Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000). However, the etiology of soybean-induced enteritis in fish remains to be elucidated.

Further processing of soybean meal into products, such as soy protein concentrate or isolate, can reduce or eliminate many of the known anti-nutritional factors in soybean meal (Krogdahl et al., 1994; Buttle et al., 2001) and reduce intestinal pathology. Processing substantially increases product cost, however, which often makes inclusion of these higher quality soybean protein sources in commercial aquaculture diets cost prohibitive, and processing may not eliminate all the anti-nutritional factors present. Additional methods of increasing soybean meal inclusion rates in soy-sensitive species, including carnivorous fish such as rainbow trout and salmon, are needed.

Probiotics have been used with varied success to treat both infectious and noninfectious enteritis in humans and other terrestrial animals (Marteau et al., 2001, 2002). Some studies suggest that probiotics function by competing with the often dietary dependent pathogenic bacteria commonly associated with the various conditions while others indicate that probiotics interact with and alter gut immune responses thus decreasing sensitization to antigens. Soy-induced enteritis responses in swine and mice have been attributed to neonatal immune development of anti-soy protein antibodies (Herman et al., 2003; Herman, 2003). Presumably, these animals get

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sensitized to soy allergens in the gastrointestinal tract and the immune system responds, and it is hypothesized that trout do the same given the similar etiology. The hindgut of teleost fish is also known to play an important immunogenic role and strong immune and inflammatory responses are achieved by delivering antigens to this site (Ellis, 1995). Similar to the results observed in terrestrial animals, microbial flora in fish has been shown to be affected by diet (Ringo and Olsen, 1999). Although the majority of previous probiotics research in aquatic animals has focused on reducing disease outbreaks (reviewed by Verschuere et al., 2000), it is difficult to attribute probiotic effects to suppression of a pathogen or other beneficial effects. The presence and endocytosis of bacteria by enterocytes in the hindgut of larvae (Hansen and Olafsen, 1999) further links the gut and its microbial flora to the development of immune responses in fish. These linkages suggest that probiotics may have the potential to alter dietary soybean antigen processing in the hindgut of first feeding fish thus altering soy sensitivity in carnivorous fish.

Rainbow trout production is a well-developed industry and a top aquaculture species in Idaho, the United States, and internationally. Due to their long propagation history, a significant amount is known about the biology, immunology, and nutritional requirements of this soy-sensitive carnivorous species (NRC, 1993), making them ideal candidates for examination of new methods to increase soy utilization. For these reasons, in the current study, rainbow trout were used to examine the ability of a commercially available dietary probiotic and graded levels of soybean meal in first feeding fry diets to increase the level of soybean meal that can be effectively utilized during a grow-out trial.

2. Materials and methods

2.1. Experimental design

To test the hypothesis that probiotic incorporation in rainbow trout starter diets containing soybean meal can induce immune-mediated soybean tolerance, a two-phase experimental design was employed. In the starter trial (first feeding,0.13 ± 0.01 to 6.5 ± 0.32 g f^{-1}), trout were fed practical type starter diets with 0, 10 or 20% soybean meal supplemented with (S0P, S10P, S20P) or without probiotics (S0, S10, S20) in a 3×2 factorial design. Potentially soy tolerant rainbow trout produced in this manner were then evaluated for their ability to utilize an industry standard level of 15% (G15) or challenge level of 43% (G43) of soybean meal during both grow-out and digestibility trials. A challenge level of 43% was chosen as the highest possible inclusion level of soybean meal at this dietary protein level. All fish handling and experimental protocols were approved by and conducted in accordance with the guidelines of the University of Idaho's Animal Use and Care Committee.

2.2. Starter experiment

First feeding fry of a domesticated strain of rainbow trout (House Creek strain, College of Southern Idaho) were utilized and a practical-type control diet that met or exceeded all the known dietary requirements of rainbow trout was formulated (NRC, 1993). The diet was formulated to contain 48% crude protein and 20% crude fat. Experimental diets were prepared with 0, 10 or 20% soybean meal (Table 1) and supplemented with or without a commercially available probiotic (Mycolactor Dry Probiotic®) obtained from Dinatec (Gainsville, GA, U.S.A.). Mycolactor Dry Probiotic® is described by the manufacturer as live, naturally occurring microorganisms with guaranteed analysis of 150 million CFU $gram^{-1}$ from *Saccharomyces cervisiae, Enterococcus faecium, Lactobacillius acidophilus, L. casei, L. plantarrum and L. brevis* and has a generally regarded as safe (GRAS) status. Mycolactor Dry Probiotic® was incorporated into the diet by replacing the cellu-fil component of the diet at the manufacturer's suggested level of 0.1%.

All ingredients were ground using an air-swept pulverizer (Jacobsen 18H, Minneapolis, MN), combined and mixed in a Marion paddle mixer prior to addition of fish oil, and pelleted using cold extrusion. A radial discharge extruder (EXDC(F)S-60) and a marumerizer (QJ-400) were used (LCI, Inc., Charlotte, NC). Thirty-two percent water was then added to the mix before extrusion through a 500 um screen. The mash was extruded at an auger speed of 19 revolutions per minute (rpm) to form wet noodles. The noodles were then placed in the marumerizer which consists of a cylindrical chamber with a rotating bottom plate. The plate is grooved to impart energy from the machine to the diet. This energy breaks the noodles, reshapes, and densifies the particles. The shaped particles were then placed in an ambient temperature (~18 °C) forced-air dryer until moisture levels were less than 10%, and stored in plastic bags at room temperature until fed.

Diets were fed to four replicate tanks of fish per treatment (300 fish $tank^{-1}$). Fish were fed to apparent satiation 6 days/week for 8 weeks. Trout were reared in 150 L tanks supplied with 4 L min^{-1} of constant temperature (14.8 °C) spring water supplied by gravity to the tanks at the University of Idaho's Hagerman Fish Culture Experiment Station. A constant photoperiod was followed (14 h daylight) using fluorescent lights controlled by a timer. Rainbow trout in the trials were bulk-weighed and counted every 3 weeks.

Table 1
Ingredients and proximate composition of starter diets containing 0, 10 or 20% soybean meal with or without probiotics for first feeding rainbow trout.[a]

Ingredients (g kg^{-1})	Diets		
	S0	S10	S20
Herring meal[b]	639.4	585.4	531.4
Soybean meal[b]	–	100.0	200.0
Wheat flour[b]	214.6	161.6	110.6
Cellu-fil or probiotic[c]	1	1	1
Fish oil[b]	111	118	123
Vitamin premix[d]	5	5	5
Lecithin[b]	20	20	20
Choline chloride[b]	6	6	6
Vitamin C[b]	2	2	2
Trace mineral mix[e]	1	1	1
Analyzed composition (±SD)[f]			
Crude protein (%)	44.3 (0.5)	44.0 (0.2)	44.4 (0.3)
Lipid (%)	17.5 (0.2)	18.3 (0.1)	18.5 (0.6)
Gross energy (kcal g^{-1})	5177 (25)	5217 (21)	5241 (26)
Ash (%)	12.5 (0.1)	11.9 (0.4)	11.9 (0.1)
Moisture (%)	5.2 (0.1)	5.0 (0.1)	4.9 (0.1)

[a] Diets were formulated on an as-fed basis.
[b] Origin of ingredients: Nelson and Sons, Murray, UT, USA.; fish oil and vitamin C were from Rangen, Buhl, ID, USA.
[c] Mycolactor Dry Probiotic® was obtained from Dinatec (Gainsville, GA, U.S.A.).
[d] Contributed per kg of diet: vitamin A (as retinol palmitate), 10,000 IU; vitamin D_3, 720 IU; vitamin E (as DL-%-tocopheryl-acetate), 530 IU; niacin, 330 mg; calcium pantothenate, 160 mg; riboflavin, 80 mg; thiamin mononitrate, 50 mg; pyridoxine hydrochloride, 45 mg; menadione sodium bisulfate, 25 mg; folacin, 13 mg; biotin, 1 mg; vitamin B_{12}, 30 ug.
[e] Contributed in mg/kg of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3.
[f] Means (±SD) of two replicate samples per diet on an as-fed basis.

2.3. Grow-out experiment

Potentially soy-tolerant fish produced as described in the starter trial remaining after sampling (described below) were pooled by starter diet, stocked into six tanks (100 fish; tank average initial tank weight 639 ± 17.5 g) per previous dietary treatment and assigned to receive one of two grow-out diets with three replicate tanks for each grow-out diet. Trout were reared in the same tanks and using the same procedures that were used in the starter experiment.

Grow-out diets were formulated to contain 41% protein and 15% lipid (Table 2). All ingredients were ground using an air-swept pulversizer (Jacobsen 18H, Minneapolis, MN). Dry ingredients were mixed in a horizontal paddle mixer and a portion (~1/3) of the added oil was mixed into the dry ingredients along with the lecithin. The

Table 2
Ingredients and proximate composition of grow-out diets containing 15 or 43% soybean meal for juvenile rainbow trout.[a]

Ingredients (g kg^{-1})	Diets	
	G15	G43
Herring meal[b]	182.2	182.2
Soybean meal[b]	150	430
Corn gluten meal[b]	210.6	34.3
Blood meal[b]	50.0	43.9
Wheat flour[b]	258.0	158.9
Fish oil[b]	119.0	124.0
Vitamin premix[c]	5	5
Lecithin[b]	20	20
Dicalcium phosphate[b]	16.2	12.7
Choline chloride[b]	6	6
Vitamin C[b]	2	2
Trace mineral mix[d]	1	1
Analyzed composition(±SD)[e]		
Crude protein (%)	42.2 (0.09)	41.8 (0.01)
Lipid (%)	14.5 (0.03)	12.7 (0.15)
Gross energy (kcal g^{-1})	5284 (1.41)	5350 (3.54)
Ash (%)	7.4 (0.01)	5.9 (0.01)
Moisture (%)	5.9 (0.09)	6.1 (0.01)

[a] Diets were formulated on an as-fed basis.
[b] Origin of ingredients: Nelson and Sons, Murray, UT, USA.; fish oil and vitamin C were from Rangen, Buhl, ID, USA.
[c] Contributed per kg of diet: vitamin A (as retinol palmitate), 10,000 IU; vitamin D_3, 720 IU; vitamin E (as DL-%-tocopheryl-acetate), 530 IU; niacin, 330 mg; calcium pantothenate, 160 mg; riboflavin, 80 mg; thiamin mononitrate, 50 mg; pyridoxine hydrochloride, 45 mg; menadione sodium bisulfate, 25 mg; folacin, 13 mg; biotin, 1 mg; vitamin B_{12}, 30 ug.
[d] Means (±SD) of duplicate replicate samples per diet on an as-fed basis.
[e] Contributed in mg/kg of diet: zinc, 37; manganese, 10; iodine, 5; copper, 3.

mash was then extruded through a 3.0 mm die of a Buhler twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland). Barrel temperature averaged 124 °C in sections 2–6, and die pressure was ~360 psi and the feed had a barrel residence time of approximately 18 s. The diets were dried in a pulse bed drier extruder (Buhler AG, Uzwil, Switzerland) with air discharge temperature remaining below 104 °C, and final moisture content less than 8%. After the diets were dried, they were top-coated with the remaining oil at ambient pressures, and stored at room temperature (~18–23 °C).

2.4. Digestibility trial

Following the end of the grow-out trial, each of the two experimental diets was modified to include yttrium oxide (100 ppm yttrium) as an inert marker for determination of apparent digestibility coefficients for protein etc. Diets were then fed for an additional 2 weeks and feces were collected by manual stripping. All fish in each tank on two separate occasions were stripped and collections were pooled for each tank. Apparent digestibility and availability coefficients for diets were calculated indirectly according to methods used by Kleiber (1961). Apparent digestibility and availability coefficients were determined according to the formula:

$$\text{ADC and AAC}(\%) = [1 - ((\%\text{IF} \times \%\text{NFs}) / (\%\text{IFsX}\%\text{NF}))] \times 100$$

where IF is the percent of indicator in the feed, NF is the percent of nutrient in the feed, IFs is the percent of indicator in the feces and NFs is the percent nutrient in the feces.

2.5. Proximate composition analyses

Dry matter and ash analysis of 10 fish $tank^{-1}$ sampled at the end of the starter and the grow-out feeding trials, diets and feces was performed according to standard methods (AOAC, 1995). Yttrium, the indigestible marker, and phosphorus were determined by inductively coupled plasma atomic absorption spectrophotometry (Perkin-Elmer Corporation, Norwalk, Connecticut, USA) following wet ashing with nitric acid (AOAC, 1995). Crude protein (N×6.25) was determined by the Dumas method (AOAC, 1995) on a LECO nitrogen analyzer (FP428, LECO Corporation, St. Joseph, Michigan, USA). Lipid was determined using a Foss Tecator Soxtec HT Solvent Extractor, Model Soxtec HT6 (Höganäs, Sweden) using methylene chloride. Total energy was determined by adiabatic bomb calorimetry (Parr 1281, Parr Instrument Company Inc., Moline, Illinois, USA).

2.6. RNA isolation and TNF-α gene expression

Intestine and liver samples from three fish per tank were isolated at the end of starter and grow-out feeding trials for RNA extraction to examine the inflammatory response marker, *TNF-α* by RT/PCR (Johansen et al., 2006). Tissue samples were immediately placed into a microtube containing TRIZol (Invitrogen, Carlsbad, CA) and RNA was isolated according to the manufacturer's protocol and then quantified. Real time RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA). Real time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA). The final concentration of each reaction was: Master Mix, 1x (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U/μL; RNase inhibitor mix, 0.4 U/μL; total RNA, 100 ng; forward and reverse primers, 900 nM for *TNF-α* and *β-actin*; and probe, 250 nM for *TNF-α* and *β-actin*. For *TNF-α* and *β-actin*, primers and probe were designed by ABI's Assay by Design service. GenbankAccession Number and Primer/Probe sequence (listed 5′-3′) for *β-actin*: AF254414, BactinF: CCCTCTTCCAGCCCTCCTT, BactinR: AGTTGTAGGTGGTCTCGTGGATA, BactinMGB: 6FAM-CCGCAAGACTCCATACCGA-NFQ; and *TNFα:* AJ401377, TNFF: TGGAGCCTCAGCTGGAGATATT, TNFR: CCGGCAATCTGCTTCAATGTATT, TNFMGB: 6FAMCATTGGTGCAAAAGATAC-NFQ. Cycling conditions for *TNF-α* and *β-actin* were as follows: 30 min at 48 °C, 10 min at 95 °C, then 40 cycles of PCR consisting of 15 s at 95 °C followed by 1 min at 60 °C. Assays were run in duplicate on RNA samples isolated from individual fish. A serial dilution of six duplicate standards was run with each primer/probe set for quantification. As a cellular mRNA control, *β-actin* levels were determined for each sample and used in the normalization of specific expression data (Kreuzer et al., 1999). The fluorescence output for each cycle of the polymerase reaction was measured and downloaded to a PC computer upon the completion of the entire run. Accumulated data was analyzed using the computer program Sequence Detector version 2.1 (Applied Biosystems, Foster City, CA). The data for *TNFα* are reported as a ratio of absolute mRNA copy number of each specific gene to the absolute copy number of *β-actin*. Ratios were multiplied by a constant variable for ease of interpretation, and expressed as means ± SE.

2.7. Histological sampling

At the end of both the starter and grow-out trials, five fish from each of the replicate tanks were randomly selected, euthanized and samples of liver and intestine were preserved in Davidson's solution for 48 h. Tissues were then transferred to 65% alcohol until processed by standard histological procedures and stained with Hematoxylin & Eosin (H&E) as described by Sheehan and Hrapchek (1983). Histological exams were conducted on distal portion of the intestine (both trials) and liver (grow-out trial only) of fingerling rainbow trout. The following criteria were used in evaluating intestinal tissue sections for the presence of enteritis: 1)widening and shortening of mucosal folds (villi) and fusion of villi, 2) abundance of absorptive vacuoles in mucosal epithelium, 3) widening of

Table 3
Growth performance[a], proximate composition[b], and intestinal TNF-α expression[c] of rainbow trout fed diets containing 0, 10 or 20% soybean meal with or without probiotics from first feeding for 8 weeks during the starter trial.

Diet	Growth			Composition				
	Weight gain[d] (% increase)	FCR[e] (g feed g gain^{-1})	Survival (%)	Moisture[b] (%)	Lipid[b] (%)	Protein[b] (%)	Energy[b] kcal g^{-1}	Intestine[b] TNF-α
Without probiotics								
0% SBM	5383a	1.00	98.5	75.5b	8.5	13.1b	6166	0.60
10% SBM	5277a	1.00	97.5	76.5a	8.2	14.3a	6114	0.77
20% SBM	4714b	0.99	97.3	77.0a	8.0	12.5c	6292	0.73
With probiotics								
0% SBM	4938	1.03	97.8	75.7	8.6a	13.8	6218	0.71
10% SBM	4959	1.02	97.1	76.7	7.8b	14.4	6191	0.64
20% SBM	5069	0.99	96.8	76.4	7.9b	13.9	6152	0.75
Pooled SE	123	0.02	1.01	0.04	0.06	0.04	24	0.09
ANOVA, Pr>F[e]	0.0142	0.8603		<0.0001	0.0016	<0.0001	0.0924	0.1413
Probiotics	0.1927	0.5749	0.5088	0.2217	0.0538	<0.0001 W>WO[f]	0.8999	0.3766
Soy	0.0401	0.5451	0.5045	<0.0001 20=10>0[f]	0.0005 0>10=20[f]	<0.0001 10>0>20[f]	0.2069	0.3527
Probiotics×soy	0.0095	0.8679	0.9923	0.0006	0.0450	<0.0001	0.0368	0.0611

[a] Means of four replicate tanks (300 fish tank^{-1}).
[b] Means of duplicate analyses of pooled sample from 10 fish per tank; four replicate tanks per diet on an as-fed basis.
[c] Final weight–initial weight/initial weight *100.
[d] FCR = feed conversion ratio; (g feed g gain^{-1}).
[e] Significance probability associated with the *F*-statistic.
[f] Explanation of statistical relationships within main factors; W=with probiotics, WO= without probiotics.

central stroma due to increased amounts of connective tissue, within mucosal folds 4) increased numbers of leucocytes (primarily lymphocytes) within the lamina propria and submucosa of the intestine, and 5) widening of the stratum granulosum along with increased numbers in the granulocytes.

2.8. Immune responsiveness and disease resistance

2.8.1. Disease challenge

At the end of the starter experiment, fish from each dietary treatment group were challenged with infectious hematopoietic necrosis virus (IHNV) to examine the effects of dietary soybean level and probiotic treatment on disease resistance. Two challenges were conducted on duplicate 25-fish groups from each dietary treatment using standard methods. Briefly, fish were challenged by waterborne exposure in 10^5 plaque forming units (PFU) mL^{-1} of IHNV strain 220-90 which has been classified as a highly virulent strain (LaPatra et al., 1994). Exposures were conducted in static baths with aeration for 1 h in a volume that was 10x the total weight of the fish in grams. Additionally, a single 25-fish mock infected control group was included for each treatment. After the exposure period, each group was placed in separate 19 L aquaria that received ultra-violet-light treated single pass spring water that was a constant 14.5 °C. Fish were fed their respective diets and were monitored for mortality for 21 days. At least 20% of the fish that died on any given day were tested for virus. Virus titers in kidney–spleen–liver homogenates were determined for some fish examined in each test. Quantitation of virus used in fish exposures or isolated from dead fish was performed by plaque assay procedures previously described (LaPatra et al., 1989).

At the conclusion of the second IHNV challenge evaluation described above, survivors from each treatment were pooled by treatment and held an additional 14 days in separate 19 L aquaria. Fish were euthanized with an overdose of tricane methane sulfonate and bled by caudal severance. Eight to 10 serum samples consisting of two or three fish from each treatment were obtained. Additionally, five serum samples of the same pool size were obtained from the mock infected control groups from each treatment. IHNV neutralizing antibody titers were determined by the complement dependent neutralization test described below.

2.8.2. Immunization trial

Following the conclusion of the grow-out feeding trial, 12 fish from each dietary treatment were anesthetized in 250 mg L^{-1} MS-222 and each fish was injected intraperitoneally with 2×10^7 PFU of an IHNV cell culture lysate. This virus isolate (039-82) has been previously shown to be significantly less virulent that other IHNV isolates. The virus was isolated, identified, and characterized as previously reported (LaPatra et al., 1994). Each 12 fish group was placed in separate 378 L fiberglass aquaria on a separate disinfected, single-pass spring water supply (mean temperature 14.5 °C). Fish were fed their respective diets daily at a rate of approximately 1% of their body weight. At 3 and 6 weeks post-immunization, fish from each treatment were anesthetized, non-lethally bled by caudal puncture and IHNV neutralization titers were determined. Additionally, five fish from the non-immunized stock groups were bled and their serum was evaluated using the same test.

To perform the complement dependent neutralization test, blood samples were allowed to clot, and then centrifuged for 10 min at 1600×*g*, and the serum was collected. Complement was inactivated in each test sample by heating the serum at 45 °C for 30 m and a two-fold dilution series was prepared. An equal volume of diluted virus (50–100 PFU) and complement (1:10 dilution of serum from disease-free rainbow trout) were then added to each serum dilution. Samples were tested in duplicate at each serum dilution by plaque assay on monolayers of the epithelioma papulosum cyprinid (EPC) cell line (Fijan et al., 1983). Anti-IHNV neutralization titers were reported as the reciprocal of the highest serum dilution that resulted in a 50% reduction in the average number of plaques detected in the negative controls (LaPata et al., 1993).

2.9. Statistics

The PROC MIXED procedure, SAS Software Version 7.00 (SAS institute, Inc., Cary, NC) was used following the starter trial to conduct a 2×3 factorial analysis of variance for a mixed effects model (Ott, 1977) in which probiotic (yes or no) and soybean inclusion level (0, 10, or 20%) were defined as a fixed effect and tank within treatments were defined as a random effect. For the grow-out trial data, an factorial analysis of variance for a mixed effects model (Ott, 1977) in which probiotic (yes or no), soybean inclusion level (0, 10, or 20%) and soybean level (15% or 43%) were defined as fixed effects and tank within treatments were

Table 4
Growth performance[a] and nutrient retention[b] of rainbow trout fed diets containing 15 or 43% soybean meal for 12 weeks during grow-out.

Starter diet	Grow-out diet	Weight gain[c] (% increase)	FCR[d] (g feed g gain^{-1})	Survival (%)	Protein retention[b]	Energy retention[b]	Phosphorus retention[b]
Without probiotics							
0% SBM	15% SBM	1515A	1.17	99	27.8	79.2	82.7
	43% SBM	1213B	1.28	98	28.5	71.9	74.3
10% SBM	15% SBM	1373A	1.21	97	28.8	77.1	86.0
	43% SBM	1341A	1.17	98	30.8	79.0	73.8
20% SBM	15% SBM	1216Bb	1.26	90yB	26.3	75.5	86.8
	43% SBM	1237Bb	1.26	94yA	30.0	71.9	68.7
With probiotics							
0% SBM	15% SBM	1387A	1.10	99	32.0	84.9	78.6
	43% SBM	1261B	1.19	98	31.8	76.9	70.2
10% SBM	15% SBM	1333A	1.18	99	29.30	78.8	98.8
	43% SBM	1367A	1.19	99	31.9	77.5	83.5
20% SBM	15% SBM	1408aA	1.19	97x	28.3	78.0	88.9
	43% SBM	1253bB	1.28	99x	28.7	72.0	69.9
Pooled SE		41	0.03	0.8	1.0	2.4	6.0
ANOVA, Pr>F[e]		0.0029	0.0126	<0.0001	0.0108	0.0268	0.1435
Probiotics		0.4884	0.0535	0.0001 W>W/O[f]	0.0143 W>W/O[f]	0.1147	0.1547
Soy		0.0658	0.0165 0=10<20[f]	<0.0001 0=10>20[f]	0.0366 0=10>20[f]	0.0474 0=10>20[f]	0.9679
Probiotics × soy		0.0969	0.1711	0.0010	0.0529	0.2697	0.4690
Grow-out Diet		0.0021 15>43[f]	0.0248 15>43[f]	0.1188	0.0148 43>15[f]	0.0065 15>43[f]	0.0017 15>43[f]
Soy × grow-out diet		0.0110	0.0593	0.0249	0.3193	0.0688	0.7053
Probiotics × grow-out diet		0.6956	0.2733	0.3415	0.3428	0.4483	0.4209
Soy × probiotics × grow-out diet		0.0404	0.4707	0.8705	0.3741	0.9268	0.7398

[a] Means of three replicate tanks (100 fish tank^{-1}).
[b] Protein retention efficiency = g crude protein fed * 100 g protein gain^{-1}; Energy retention efficiency =kcal energy fed*100 kcal energy gain^{-1}; Phosphorus retention efficiency= g phosphorus fed * 100 g^{-1} phosphorus gain.
[c] Final weight–initial weight/initial weight *100.
[d] FCR = feed conversion ratio; (g feed g gain^{-1}).
[e] Significance probability associated with the *F*-statistic.
[f] Explanation of statistical relationships within main factors; W=with probiotics, WO= without probiotics.

defined as a random effect. Binomial data was transformed using the arcsine transformation before analysis. Differences among treatment means ($n=4$ t trt^{-1} starter trial and $n=3$ t trt^{-1} grower trial) were determined using the Tukey procedure for pair-wise comparisons (Tukey, 1953). The Wilcoxon Rank-Sum test and the Mann–Whitney *U* test were used to make comparisons between antibody titers. Treatment effects in all statistical analyses in this project will be considered different when probabilities for a greater *F* value are less than 0.05.

3. Results

3.1. Growth performance

3.1.1. Starter trial; survival and proximate composition

Dietary soybean meal level and probiotic supplementation significantly altered weight gain of first feeding rainbow trout (Table 3). Specifically, a decrease in growth was observed for fish fed S20 but not for fish fed the same diet with probiotics (S20P). In contrast, no significant effects or interactions were observed among dietary treatments for feed conversion ratio or survival (Table 3).

There was a significant effect of soybean level in starter diet on whole body proximate composition (Table 3). Fish fed S10 and S20 had decreased lipid content and increased moisture when compared to fish fed the S0 diet. Feeding diets with probiotic supplementation exacerbated this effect (Table 3). However, these differences did not result in altered energy content of the whole body. A significant interaction between dietary soybean level and probiotic supplementation was also observed for whole body protein. Fish fed S20 had lower whole body protein than all other treatments (Table 3).

3.1.2. Grow-out trial; growth, survival and nutrient retention

Growth performance was significantly altered by grow-out diets and previous dietary history (starter diet); significant interactions

Table 5
Percent protein, lipid, and energy apparent digestibility coefficients (ADC) and phosphorus apparent availability coefficient (AAC) values of diets containing 15 or 43% soybean meal fed to juvenile trout at the conclusion of the grow-out trial.[a]

Starter diet	Grow-out diet	Crude protein ADC	Crude lipid ADC	Gross energy ADC	Phosphorus AAC
Without probiotics					
0% SBM	15% SBM	88.3	97.4	81.4	51.6
	43% SBM	85.5	92.6	72.2	66.9
10% SBM	15% SBM	86.9	97.4	79.0	54.3
	43% SBM	83.9	94.3	74.2	66.3
20% SBM	15% SBM	88.2	97.9	81.3	51.5
	43% SBM	85.1	93.3	73.3	67.2
With probiotics					
0% SBM	15% SBM	89.0	98.0	82.0	52.3
	43% SBM	84.3	94.8	74.7	64.9
10% SBM	15% SBM	87.6	97.0	80.5	51.4
	43% SBM	85.5	94.2	73.5	67.4
20% SBM	15% SBM	87.3	96.3	79.3	48.2
	43% SBM	82.3	91.3	67.9	64.9
Pooled SE		2.1	1.0	3.8	2.2
ANOVA, Pr > F[b]		0.5349	0.0003	0.2155	<0.0001
Probiotics		0.7966	0.5734	0.7935	0.2613
Soy		0.7702	0.2247	0.7316	0.4712
Probiotics × soy		0.6180	0.0874	0.5911	0.7433
Grow-out II diet		0.0097 15>43[c]	<0.0001 15>43[c]	0.0013 15>43[c]	<0.0001 15>43[c]
Soy × grow-out diet		0.8843	0.4623	0.7754	0.6971
Probiotics × grow-out diet		0.6925	0.6940	0.7846	0.7757
Soy × probiotics × grow-out diet		0.8677	0.7604	0.8813	0.5431

[a] Means of three replicate tanks.
[b] Significance probability associated with the *F*-statistic.
[c] Explanation of statistical relationships within main factors.

Table 6
TNF- α mRNA expression in livers and intestine of rainbow trout fed diets containing 15 or 43% soybean meal at the conclusion of the grow-out trial.[a]

Starter diet	Grow-out diet	Liver TNF-α	Intestine TNF-α
Without probiotics			
0% SBM	15% SBM	1.31	0.68
	43% SBM	2.12	1.43
10% SBM	15% SBM	1.18	2.47
	43% SBM	2.21	2.18
20% SBM	15% SBM	2.19	1.88
	43% SBM	1.42	1.03
With probiotics			
0% SBM	15% SBM	1.59	1.90
	43% SBM	1.77	1.28
10% SBM	15% SBM	1.06	0.85
	43% SBM	3.81	3.26
20% SBM	15% SBM	1.36	1.66
	43% SBM	4.23	2.74
Pooled SE		0.68	0.79
ANOVA, Pr>F[b]		0.0021	0.4903
Probiotics		0.1602	0.2713
Soy		0.4678	0.5430
Probiotics × soy		0.4629	0.5182
grow-out diet		0.0069	0.3754
		43>15	
Soy × grow-out diet		0.3790	0.1061
Probiotics × grow-out diet		0.0776	0.4467
Soy × probiotics × grow-out diet		0.1222	0.7614

[a] Means of three replicate tanks.
[b] Significance probability associated with the *F*-statistic.
[c] Explanation of statistical relationships within main factors.

between grow-out diet and starter diet were observed (Table 4). Fish fed S10 had equivalent growth when fed G15 or G43, while fish fed S0 or S20 had lower growth when fed G43. Fish fed S20 displayed reduced growth when fed G15 or G43. Fish fed S20 had significantly higher FCRs at the end of grow-out trial as compared to fish fed S0 or S10. Fish fed G43 had significantly higher FCRs than fish fed G15 regardless of starter diet. Fish fed S20 displayed reduced survival regardless of grow-out diet.

Similarly, nutrient retention was altered by dietary treatment (Table 4). Fish fed G43 had significantly decreased phosphorus retention regardless of starter diet. Significant effects of starter diet were observed for protein and energy retention. Feeding probiotics during the starter trial increased protein retention in grow-out while feeding S20 decreased grow-out protein retention. Fish fed G43 had slightly higher, albeit significant, protein retention than fish fed G15. Feeding S20 decreased grow-out energy retention. Fish fed G43 had significantly higher energy retention that fish fed G15.

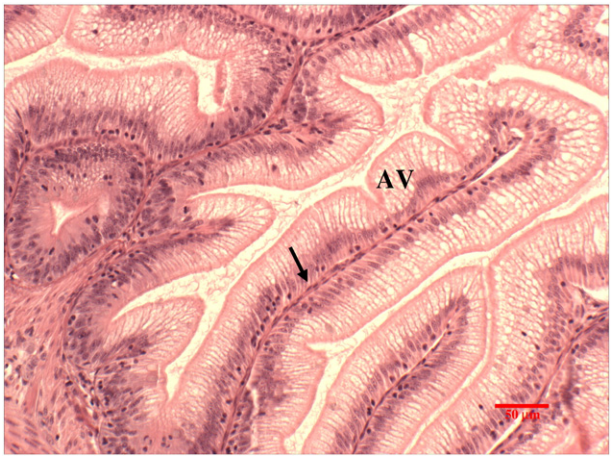

Fig. 1. Mucosal epithelium of control fish fed S0 during the starter trial contains numerous, finely granular absorptive vacuoles (AV). Connective tissue stroma is scant in the lamina propria within the center of villi (arrow). Bar = 50 µm.

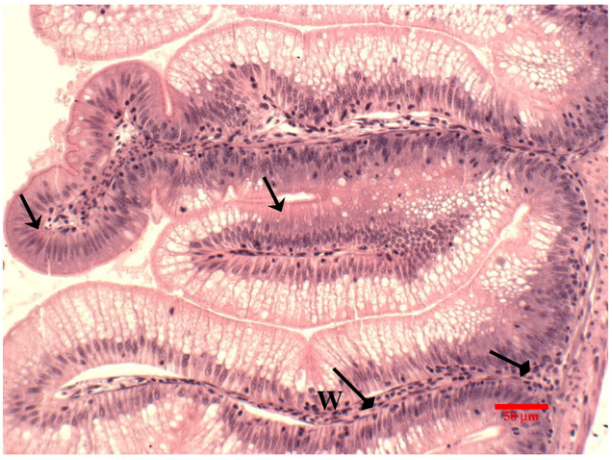

Fig. 2. Absorptive vacuoles are lacking in mucosal epithelial cells in villi of fish fed S20 during the starter trial. Note also slight increase in numbers of lymphocytes in lamina propria (diamond arrows) and widening (W) of central stroma. Bar = 50 µm.

3.1.3. Digestibility data

Apparent digestibility coefficients (ADC) for protein, lipid and energy and phosphorous apparent availability coefficient (AAC) were significantly altered by grow-out diet (Table 5). Fish fed G43 had significantly decreased protein, lipid and energy ADC and significantly increased phosphorus AAC when compared to fish fed G15 diet regardless of starter diet.

3.2. TNF-α expression

No significant effects of diet on intestinal TNF-α expression were observed at the end of the starter or grower trial (Table 6). In contrast, liver TNF-α expression at the end of the grow-out trial was significantly altered by grow-out diet (Table 6). Fish fed G43 had significantly higher TNF-α in the liver than fish fed G15.

3.3. Histology

3.3.1. Starter trial histology

Feeding increased levels of soybean meal during the starter trial substantially altered intestinal tract histology. Fish fed diets containing

Fig. 3. Normal villi from fish fed S0/G15. Mucosal epithelium is indicative of normal absorptive vacuoles (AV), some of which are distended. Note thin central stroma of villi contains few lymphocytes (arrow). Stratum granulosum is thin and contains few eosinophilic granulocytes (large arrow). Bar = 50 µm.

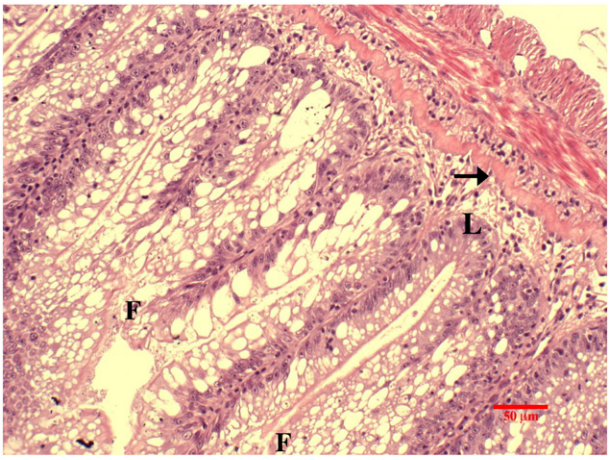

Fig. 4. Fusion of villi (F), cystic absorptive vacuoles in mucosal epithelium, inflammation of lamina propria (L) and increased numbers of eosinophilic granular cells in stratum granulosum (arrow). Central stroma of villi is thickened and inflamed in fish fed S0/G43. Bar = 50 µm.

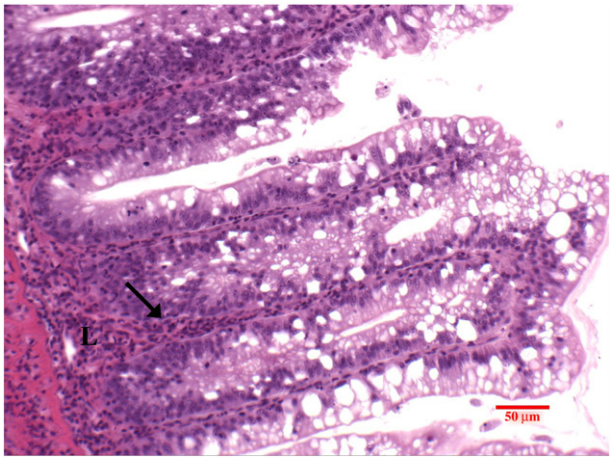

Fig. 6. Fusion of three villi in fish fed S0/G43. Absorptive vacuoles are cystic and central stroma is thickened and inflamed (arrow) as is lamina propria (L). Bar = 50 µm.

S20 had fewer absorptive vacuoles in mucosal epithelial cells than those fed S0 or S10 (Figs. 1 and 2). There was no noticeable difference in amount of absorptive vacuoles when comparing intestines of fish fed S20 and S20P. In addition, focal areas lacking absorptive vacuoles were seen more frequently in mucosal epithelium of fish fed S20 (Fig. 2) than in fish fed other diets. There was a slight increase in numbers of lymphocytes within the lamina propria and submucosa of fish fed S20 when compared to those fed S0 and S10 (Fig. 2). There was no difference, however, in numbers of intestinal lymphocytes between fish fed S20 or S20P. Central stroma within villi was also slightly increased in fish fed diets containing S20 when compared to those of fish fed S0 and S10 (Fig. 2). However, there was very little difference in the amount of central stroma between fish fed S20 and S20P.

All other evaluation criteria showed minimal effects due to diet. Specifically, there was no noticeable difference in the width of the intestinal stratum granulosum due to diet. Widening and shortening of villi were not apparent in descending intestines of fish fed all test diets when compared with those of controls. Mild fusion of villi was present to a certain extent in intestines of all fish including controls.

3.3.2. Grow-out trial histology

Liver histology was normal and indicated no significant effect of dietary treatment. Cytoplasmic vacuolation of hepatocytes, indicative of glycogen/fat storage, varied from mild to moderate in all treatments. In contrast, substantial effects of dietary treatments were observed for intestinal histology. Descending intestines of fish fed S0/G15 and S0P/G15 appeared mostly normal. Large vacuoles were sometimes seen in mucosal epithelial cells (Fig. 3). Significant pathological changes were seen in intestines of fish fed S0/G43 and S0P/G43. These changes consisted primarily of fusion of villi, leucocytc inflammation of the lamina propria and increased numbers of granular cells of the stratum granulosum (Fig. 4). In addition, the central stroma of villi was often thickened and inflamed. Absorptive vacuoles of mucosal epithelial cells were often cystic in nature and lacked the fine granularity of those seen in fish fed G15 (Fig. 4). Nuclei of mucosal epithelial cells varied from columnar to cuboidal and were often pelomorphic. Necrosis and sloughing of mucosal epithelial cells was also noted (Fig. 5). Histologically, intestinal tissue of fish fed S0/G15 and S0P/G15 was also normal while significant pathological changes were seen in intestines of fish fed S0/G43 and S0P/G43 (Figs. 6 and 7).

Histological examination of descending intestines of fish fed S10/G15 and S10P/G15 showed tissues that were mostly normal. Changes in histological structure of fish fed the S10/G43 and S10P/G43 were similar to those changes described in the intestines of fish S0/G43 and S0P/G43. However, these changes did not appear to be as severe as that seen in fish fed S0.

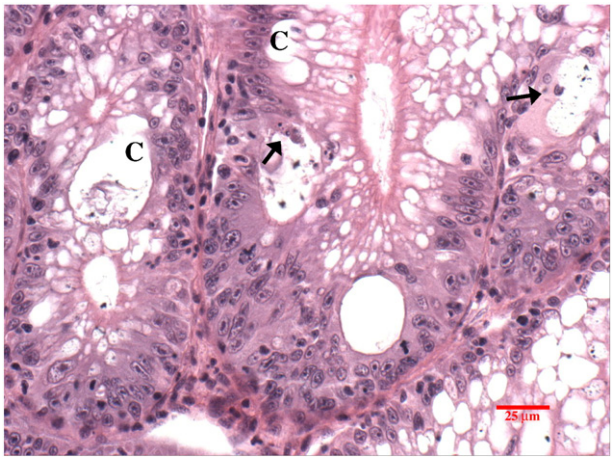

Fig. 5. Coalescence of absorptive vacuoles of mucosal epithelium into cystic spaces (C) in fish fed S0/G43. Some contain degenerate epithelial cells (arrows). Note variation in nuclear size and shape of mucosal epithelium. Bar = 25 µm.

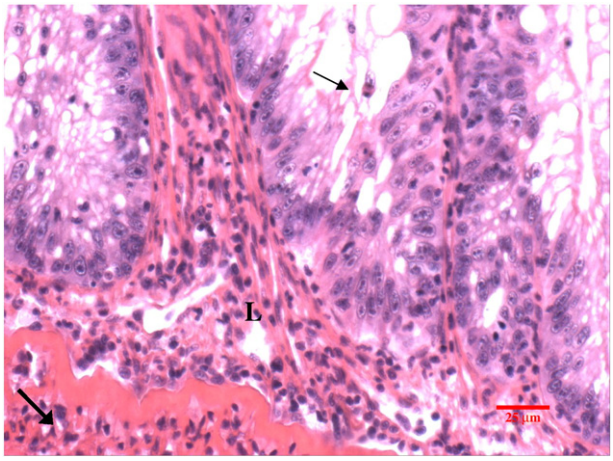

Fig. 7. Leucocytic inflammation of lamina propria (L) and well as an increase in thickness of the stratum granulosum (arrow) due to increased numbers of eosinophilic granular cells in fish fed the S0P/G43. Mucosal epithelial cells have cystic AV's and pleomorphic nuclei. See also necrotic sloughed cell (thin arrow). Bar = 25 µm.

Histological examination of descending intestines of fish fed S20/G15 and S20P/G15 displayed small focal areas lacking absorptive vacuoles in the primary villi (folds), but this was not common. Absorptive vacuoles were generally normal with fine granularity but some were slightly swollen. Focal areas of mild inflammation were occasionally seen in the lamina propria. Histological changes of fish fed S10/G43 and S20/G43 appeared to be slightly less severe than those fish previously fed S0/G43, however, and in one fish intestinal tissue was mostly normal. Absorptive vacuoles in mucosal epithelium of fish fed S20/G43 were less cystic than seen in intestines of fish fed S0/G43 or S10/G43.

3.4. IHNV resistance and immunization trials

There was no significant effect of soybean meal or probiotics inclusion on survival following two separate IHNV challenges. Survival averaged 62% for fish fed S0, 54% for fish fed S10 and 62% for fish fed S20 in the first trial ($P=0.8567$) and 72%, 71%, and 63%, respectively in the second trial ($P=0.4550$). However, circulating antibody titers detected in the survivors of the second challenge indicate that fish fed S20 or S20P and S10P had higher titers than surviving fish fed S0 or S0P or S10 (data not shown).

Similarly, only minor effects of dietary treatment were observed following IHNV immunization at the end of the grow-out trial. Regardless of grow-out diet, fish fed S0 or S10 had generally higher titers at 6 weeks than at 3 weeks, while fish fed S20 generally had higher titers at 3 weeks than at 6 weeks. Of note, fish fed S10P/G15 had generally low titers at each time point (data not shown).

4. Discussion

The use of bacterial preparations to adapt fish to new types of nutrients represents a novel probiotic application that was proposed by Heikkinen et al. (2006). In that study, Heikkinen et al. (2006) determined that inclusion of soybean meal in the diet of juvenile rainbow trout substantially altered both number and diversity of intestinal bacterial species, indicating that it might be possible to colonize fish intestines with bacteria that could aid the host in adjusting to dietary changes. Probiotic mechanism(s) are the subject of ongoing debate and likely multifactoral including production of anti-bacterial inhibitory compounds and competition for chemicals and adhesion sites leading to an "improved" microbial balance as well as host immune modulation and modification of dietary components to increase utilization by the host (Verschuere et al., 2000). The latter two mechanisms are particularly relevant to soybean meal utilization in carnivorous fish given the observed detrimental effects on rainbow trout growth and immune response commonly associated with anti-nutritional and antigenic factors present in soybean meal. Therefore, in the current study, we tested the hypothesis that probiotics can alter soy sensitivity in rainbow trout by first attempting to develop soy tolerant rainbow trout and then conducting a soy challenge to determine if soy sensitivity had been decreased.

A beneficial effect of probiotic supplementation on growth of fish fed 20% soybean meal was observed at the end of the starter trial, seemingly validating Heikkinen's hypothesis. However, no detectable benefit of probiotic supplementation was observed in fish fed 20% soybean meal on gut health or inflammatory status as measured intestinal TNF-α expression. Kim and Austin (2006) observed a similar lack of responsiveness of intestinal TNF-α expression in rainbow trout cells co-cultured with probiotics. Stimulation of the immune system is considered an important mechanism to support probiotic activity (Hong et al., 2005; Irianto and Austin, 2002; Panigrahi et al., 2004). However, in the current study no benefit of feeding the putative probiotic on survival was observed following controlled challenge with IHNV. In contrast, a beneficial effect of probiotic was observed for fish fed 20% soybean meal on whole body protein composition in the current study. Macey and Coyne (2005) showed that probiotics enhanced the growth rate of abalone by improving protein digestion and absorption of the digestive tract thus a similar effect may have occurred in the present study. An important feature of putantive probiotics is the ability to colonise fish gut (Nikoskelainen et al., 2001). Given that the probiotic chosen for our study was designed to enhance soybean meal utilization by swine not cold water aquatic species, it is unclear if the results observed in the current study are due to colonization.

Significant differences in gut health also were observed due to dietary soybean meal level during the starter trial. Fish fed 20% soybean meal had decreased growth and numbers of absorptive vacuoles relative to fish fed 0 or 10% soybean meal regardless of probiotic supplementation. The number of vacuoles in enterocytes has been suggested by Ezeasor (1978) to be indicative of protein and lipid absorption capability of the rainbow trout posterior intestine. However, fish fed the S10 grew equivalently to fish fed S0 in the current study suggesting adequate absorptive capacity and questioning the sensitivity of this index. The histology data does, however, demonstrate a soy dependent response on gut health similar to that previously observed for juvenile trout (Heikkinen et al., 2006; Ostaszewska et al., 2005) even though the highest level of soybean meal utilized in the present study was 20% while the previous studies inclusion levels were 44 and 45%, respectively. It is generally accepted that low to moderate levels of soybean meal may be included in salmonid diets without serious negative effects on growth or feed utilization (Krogdahl et al., 2000; Refstie et al., 2000) but inflammatory responses of the gut will be present (Baeverfjord and Krogdahl, 1996).

The practical application of probiotics to improve soy utilization in trout fry diets at the levels examined in starter trial are limited given that most salmonid fry diets currently do not contain soybean meal. More importantly, is whether the inclusion of probiotics and/or soybean meal in the diet of first feeding trout altered soy sensitivity in a manner that would increase utilization when these fish are fed higher levels during the grow-out portion of the production trial. Growth performance of trout during grow-out supports a role of dietary history in soy sensitivity; both soy and probiotic inclusion in starter diets affected fish performance in grow-out. The probiotic effects observed during grow-out, however, may simply indicate an inability of fish fed S20 to overcome existing tissue damage. The existing damage could then result in a failure to thrive and decreased survival regardless of soybean level in grow-out diets rather than a persistent beneficial effect of probiotics in fish fed S20P that would be indicative of colonization.

Previous research indicates that at levels of >40% reduced weight gain and feed efficiency (Olli and Krogdahl, 1995; Olli et al., 1995; Rumsey et al., 1994) and pathomorphological changes in the distal intestinal epithelium (Baeverfjord and Krogdahl, 1996; Rumsey et al., 1994; Van den Ing et al., 1991) are observed. Thus, the lack of growth suppression in fish fed S10/G43 could indicate an adaptation or reduced sensitivity to soybean meal. Lending support for this idea is the histological data which suggests less severe intestinal damage in fish fed S10/G43. However, this purported adaptation did not correlate to improved digestibility values in fish fed the G43 diets or to decreased inflammatory response as measured by liver TNF-α expression. Liver TNF-α expression was increased in fish fed G43 in the absence of distinguishable liver pathology suggesting a more global effect of dietary soybean meal on inflammatory or immune status. However, this global effect was not detected by the immunization trial at the end of the grow-out trial. Previous research has shown an association between sub-acute enteritis with inferior nutrition in soy-fed rainbow trout and elevated immune responses (Rumsey et al., 1994) while dietary soybean meal at dietary inclusion rates of >30% has been shown to suppress immune capacity (Burrells et al., 1999; Rumsey et al., 1994; Krogdahl et al., 2000).

For most response variables a level of 43% soybean meal during grow-out significantly reduced performance regardless of starter diet, with the exception of the protein retention. This protein retention effect may be explained by the slightly higher lysine content in the G43 diet (2.73) as compared to the G15 diet (2.10), although both diets met the published lysine requirements for rainbow trout (1.80; Hardy, 2002).

5. Conclusions

Feeding diets with increased levels of plant protein and reduced levels of fish meal, has many benefits. Soybean meal is readily available and cost competitive, but dietary levels are limited due to deleterious effects on growth. The results of this study indicate that the amount of dietary soybean meal tolerated by rainbow trout can be increased in the starter trial of production by feeding probiotics. Dietary soybean meal levels may also be increased in grow-out diets by inclusion of low levels of soybean meal in the starter diets. Feeding diets with higher levels of soybean meal and lower levels of fish meal can decrease feed costs and increase sustainability of production and reduce dependence on marine harvest.

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